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Hikeshi, a Nuclear Import Carrier for Hsp70s, Protects Cells from Heat Shock-Induced Nuclear Damage

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SUMMARY

During heat shock stress, importin β family-mediated nucleocytoplasmic trafficking is downregulated, whereas nuclear import of the molecular chaperone Hsp70s is upregulated. Here, we identify a nuclear import pathway that operates during heat shock stress and is mediated by an evolutionarily conserved protein named "Hikeshi," which does not belong to the importin β family. Hikeshi binds to FG-Nups and translocates through nuclear pores on its own, showing characteristic features of nuclear transport carriers. In reconstituted transport, Hikeshi supports the nuclear import of the ATP form of Hsp70s, but not the ADP form, indicating the importance of the Hsp70 ATPase cycle in the import cycle. In living cells, depletion of Hikeshi inhibits heat shock-induced nuclear import of Hsp70s, reduces cell viability after heat shock stress, and significantly delays the attenuation and reversion of multiple heat shock-induced nuclear phenotypes. Nuclear Hsp70s rescue the effect of Hikeshi depletion at least in part. Thus, Hsp70s counteract heat shock-induced damage by acting inside of the nucleus.

INTRODUCTION

Regulation of the nucleocytoplasmic transport of macromolecules through nuclear pore complexes (NPCs) is crucial for various cellular functions in eukaryotic cells. Members of the importin β family, referred to as importins, transportins, exportins, or karyopherins, are the best-characterized nucleocytoplasmic carrier molecules and are thought to mediate most of the selective nucleocytoplasmic protein transport (Görlich and Kutay, 1999; Weis, 2003; Stewart, 2007). In human cells, more than 20 members of the importin β family carrier proteins have been identified. Importin β family carriers interact with their specific cargos, components of the NPC (nucleoporins [Nups]), and the small GTPase Ran. Interactions with Nups allow importin β family carriers to shuttle continuously between the cytoplasm and the nucleus. All of the importin β family carriers' transport pathways couple with the GTPase cycle of the small GTPase Ran, which is mediated by chromatin-bound guanine nucleotide exchange factor regulator of chromosome condensation 1(RCC1) (RanGEF) and cytoplasmic GTPase-activating protein RanGAP (Görlich and Kutay, 1999; Weis, 2003; Stewart, 2007). Because the binding of RanGTP to transport carriers regulates cargo release and loading, the sharp concentration gradient of RanGTP that exists at the boundary of the nuclear envelope (high in the nucleus and low in the cytoplasm) (Kaláb et al., 2006) allows cargo to accumulate in one compartment against the chemical concentration gradient.

Cellular stresses affect many aspects of cellular physiology, including the dynamic redistribution of nucleocytoplasmic localization of various proteins. One notable phenomenon is the perturbation of conventional nuclear import pathways. Stresses, such as heat shock and oxidative stress, induce nuclear retention and nuclear export inhibition of importin α, which is the classical nuclear localization signal (NLS) receptor, resulting in suppression of the importin α- and β-dependent import pathway (Furuta et al., 2004; Miyamoto et al., 2004). More seriously, these cellular stresses have been reported to induce perturbation of the RanGTP gradient between the nucleus and the cytoplasm (Czubryt et al., 2000; Furuta et al., 2004; Kodiha et al., 2004; Miyamoto et al., 2004; Kelley and Paschal, 2007), which leads to downregulation of all importin β family-mediated pathways, suggesting that these pathways are unlikely to operate in cells under stress conditions.

A shift in the temperature of an organism from the physiological state causes protein misfolding, protein dysfunction, or unspecific aggregation (Richter et al., 2010). This protein damage occurs in various cellular compartments, leading to lethal effects. To maintain and restore protein homeostasis, many molecular chaperones are rapidly expressed in response to environmental stresses. Hsc70/Hsp70 (Hsp70s) belong to a major family of molecular chaperones involved in highly conserved protective systems (Kampinga and Craig, 2010; Mayer, 2010). Hsp70s generally function with two groups of co-chaperones. The first is a group of Hsp40, J domain-containing proteins that stimulate the hydrolysis of ATP Hsp70s, and the second group includes nucleotide exchange factors (NEFs) such as Bag and Hsp110 families. The nucleotide state of Hsp70s is crucial for the binding and release of their substrates, such as nonnative proteins. In response to heat shock, Hsp70s are rapidly and transiently relocated from the cytoplasm into the nucleus and nucleolus by an as-yet unknown mechanism

(Lamian et al., 1996; Pelham, 1984; Velazquez and Lindquist, 1984; Welch and Feramisco, 1984).

Numerous nuclear events, including ribosome biogenesis, DNA metabolism, and RNA transcription or processing, have been described as stress sensitive. These nuclear events frequently accompany significant changes in the organization or components of nuclear structures. Such stress-induced nuclear phenotypes must be reverted during recovery from stress for cells to survive. However, the mechanisms underlying the reversion of the stress-induced nuclear phenotypes are very poorly understood. Moreover, the nuclear functions of Hsp70s during heat shock stress have not been well studied, despite the fact that their nuclear accumulating property in response to heat shock has been documented for about 30 years.

Here, we report the identification of a nuclear import carrier for the heat shock-induced nuclear import of Hsp70s. The carrier is an evolutionarily conserved protein with unknown function. This protein is encoded by chromosome 11 open reading frame 73 (the *C11orf73* gene) and does not belong to the well-characterized importin β family. The product of *C11orf73* binds directly to ATP-form Hsp70s and dissociates preferably from ADP-form Hsp70s, indicating that carrier-cargo binding and release are regulated by the Hsp70s ATPase cycle. Consistently, cochaperones that regulate the ATPase cycle of Hsp70s play crucial roles in the nuclear import of Hsp70s. Furthermore, C11orf73 binds directly to phenylalanine-glycine (FG) repeat-containing nucleoporins (FG-Nups) and translocates through NPCs, like all known nuclear transport carriers. These findings show that the mechanism of import mediated by C11orf73, such as the force driving the import, is different from the importin β family-mediated pathway, which is coupled with GTPase cycle of RanGTP. Moreover, we show that the C11orf73-mediated nuclear import pathway is important for the reversion and attenuation of multiple heat shock-induced nuclear phenotypes and therefore the protection of cells from heat shock damages. We thus named this carrier "Hikeshi," which is a traditional Edo-era Japanese compound word used for a firefighter, smokejumper, or troubleshooter. Our results demonstrate that Hikeshi-mediated nuclear import of Hsp70s is required, at least in part, for protection of cells from heat shock damage and that Hsp70s counteract the damage by acting inside the nucleus.

RESULTS

In Vitro Reconstitution of Heat Shock-Induced Nuclear Import

During heat shock stress, the conventional nuclear import pathway is downregulated (Furuta et al., 2004; Miyamoto et al., 2004), whereas the nuclear import of molecular chaperone Hsp70s is upregulated (Pelham, 1984; Velazquez and Lindquist, 1984; Welch and Feramisco, 1984). To dissect the molecular mechanism of the heat shock-induced nuclear import of Hsc70, we first set up an experimental system that mimics nuclear import in living cells observed during heat shock stress by using permeabilized cells. For this, we prepared cytosol and permeabilized cells from normal or heat shock-treated HeLa cells and performed transport assays with four different combinations (heat-shocked cytosol versus normal or heat-shocked permeabilized cells, or normal cytosol versus normal or heat-shocked permeabilized cells; see Figure 1A) by incubating with recombinant GFP-Hsc70 proteins in the presence of ATP and its regeneration system. With this approach, we found that efficient nuclear accumulation of Hsc70 was supported only by cytosol extracted from heat-shocked cells (Figure 1B). The import was unaffected by the source of the permeabilized cells;

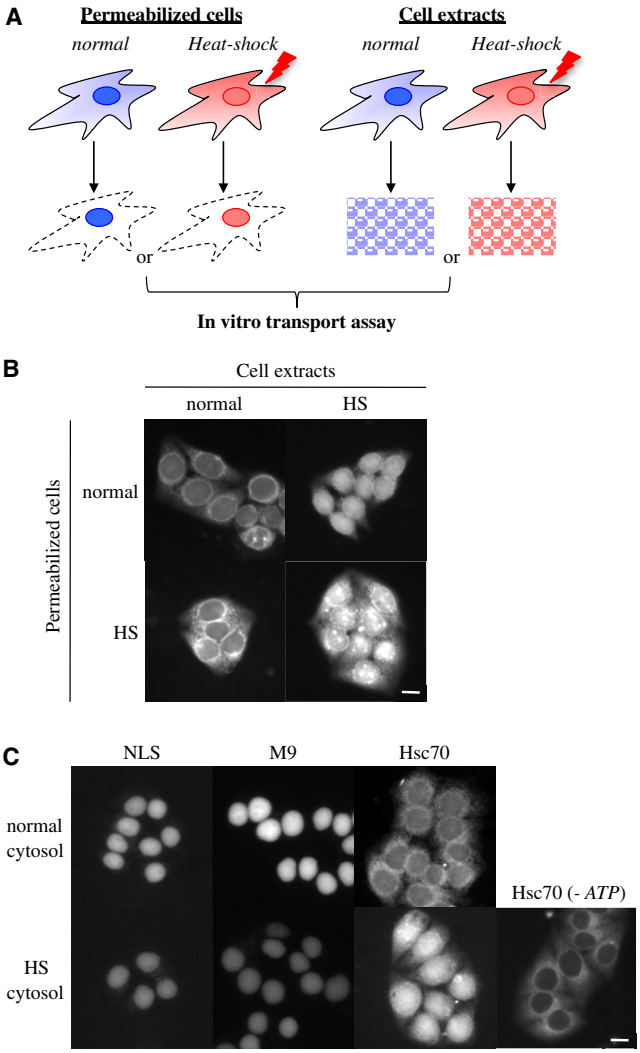


Figure 1. In Vitro Reconstitution of Heat Shock-Induced Nuclear Import

(A) Permeabilized cells and cell extracts for in vitro transport assays were prepared from either normal or heat-shocked HeLa cells.

(B) Recombinant GFP-Hsc70 protein was incubated with the permeabilized cells and cell extracts, prepared as in (A), in the presence of ATP. Scale bars, 20 μm.

(C) Nuclear transport assays were performed using HeLa cytosol prepared from heat shock-treated or nontreated cells and ATP. NLS cargo and M9 cargo are imported by two members of the importin β family: importin β and transportin, respectively. Cytosol prepared from heat shock-treated cells does not efficiently mediate conventional import pathways facilitated by the importin β family but does mediate the nuclear import of Hsc70. The nuclear import of Hsc70 is also dependent on the presence of ATP. Scale bar, 20 μm.

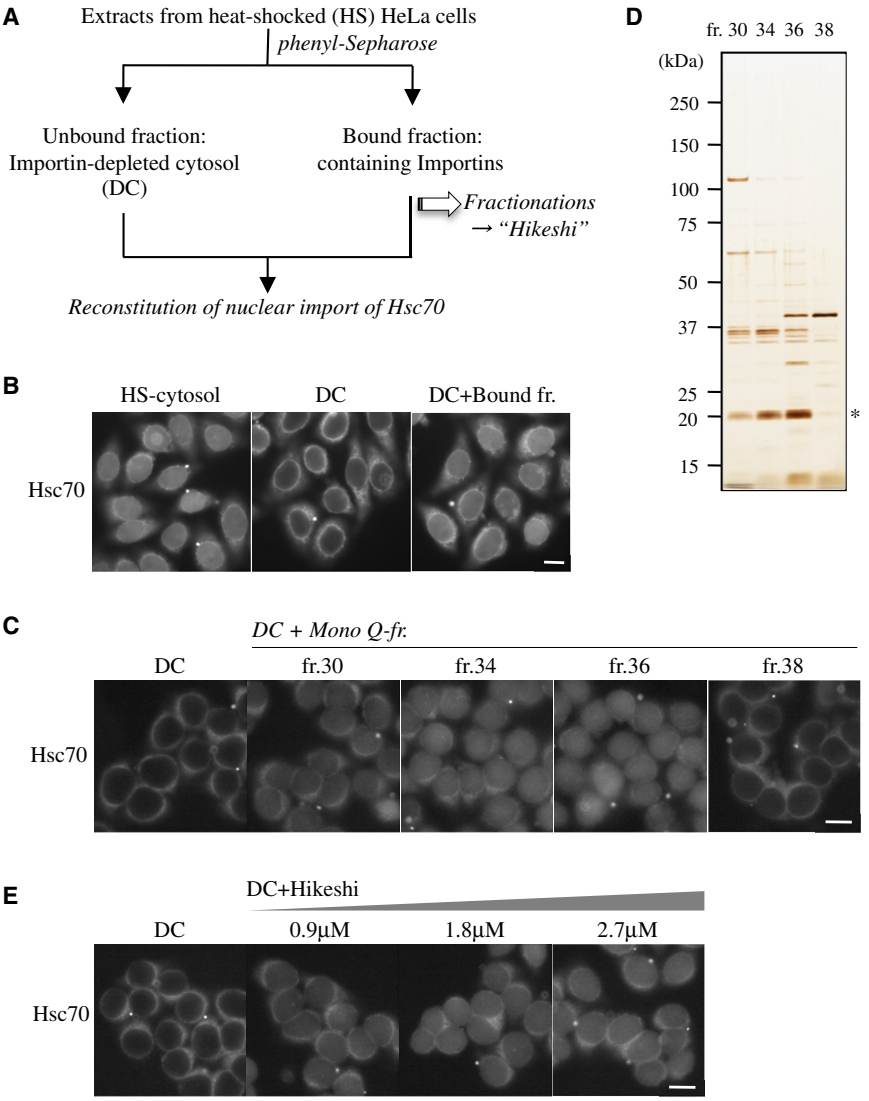


Figure 2. Identification of Hikeshi as an Essential Protein for the Nuclear Import of Hsp70s

(A) Reconstitution scheme for the heat shock-induced nuclear import of Hsc70.

(B) Importin-depleted cytosol (DC) does not support the nuclear import of recombinant GFP-Hsc70 protein in permeabilized cells, showing that the nuclear import factor of Hsc70 is absorbed by phenyl-Sepharose from heat-shocked total extracts (Figure S1). Scale bar, 20 μm.

(C) Addition of the final MonoQ fractions to DC facilitates the nuclear import of GFP-Hsc70 in permeabilized cells. Scale bar, 20 μm.

(D) Protein profiles of final MonoQ fractions. (Asterisk) Hikeshi bands support the nuclear import activity of Hsc70 (Figure 2C).

(E) Hikeshi facilitates the nuclear import of Hsc70. Permeabilized cells were incubated with increasing concentrations of purified recombinant Hikeshi protein, as indicated, in the presence of DC and ATP for 20 min at 30°C. Scale bar, 20 μm. See also Figure S1.

heat shock-induced nuclear import was sufficiently reconstituted with permeabilized cells prepared from normal cells or heat-shocked cells. Notably, the nuclear import of Hsc70 was inhibited strongly in the absence of ATP, suggesting a strict requirement for an energy source (Figure 1C). In the same setting, the conventional nuclear import mediated by importin β and transportin was suppressed (Figure 1C), showing that the reconstituted nuclear import mimics reactions observed in heat-shocked living cells.

Identification of Hikeshi as an Essential Protein for Nuclear Import of Hsp70s

Nuclear transport carriers are thought to have hydrophobic properties necessary to interact with and translocate through NPCs and are enriched by phenyl-Sepharose under stringent binding conditions (Ribbeck and Görlich, 2002). We applied this approach to identify the cytosolic factor(s) required for the nuclear import of Hsc70. Cytosol prepared from heat-shocked cells was separated into importin-containing and importin-depleted fractions using a phenyl-Sepharose column (Figure 2A and Figure S1A available online). As shown in Figure 2B, the nuclear import of Hsc70 was not supported by the importin-depleted fraction but was reconstituted when the importin-containing fraction was supplemented to the depleted fraction. To examine whether importin β family members can restore the nuclear import of Hsc70, we added each individual recombinant importin to the importin-depleted fraction and then examined the import activity. No importin, alone or in various combinations, restored the nuclear import of Hsc70 (Figure S1B).

Thus, the nuclear import of Hsc70 under heat shock conditions seems unlikely to be mediated by importins but requires a soluble factor or factors absorbed by the phenyl-Sepharose column. We attempted to identify the factor or factors by further biochemical purification based on Hsc70 import activity from the importin-containing fraction. After the final column step, one candidate protein was obtained that showed a good correlation with the import activity of Hsc70 (Figures 2C and 2D). Liquid chromatography tandem mass spectrometry (LC-MS/MS) analysis revealed that this protein was a product of human *C11orf73* (chromosome 11 open reading frame 73) gene (Gene ID: 51501), which we renamed Hikeshi based on its cellular functions described below. The gene encodes an uncharacterized 197 amino acid protein, which is highly conserved from yeast to humans (Figure S1C) (Fernández-Valdivia et al., 2006; Hancock et al., 2006). When bacterially expressed purified recombinant Hikeshi was added to the importin-depleted fraction, the nuclear import of Hsc70 was restored (Figure 2E).

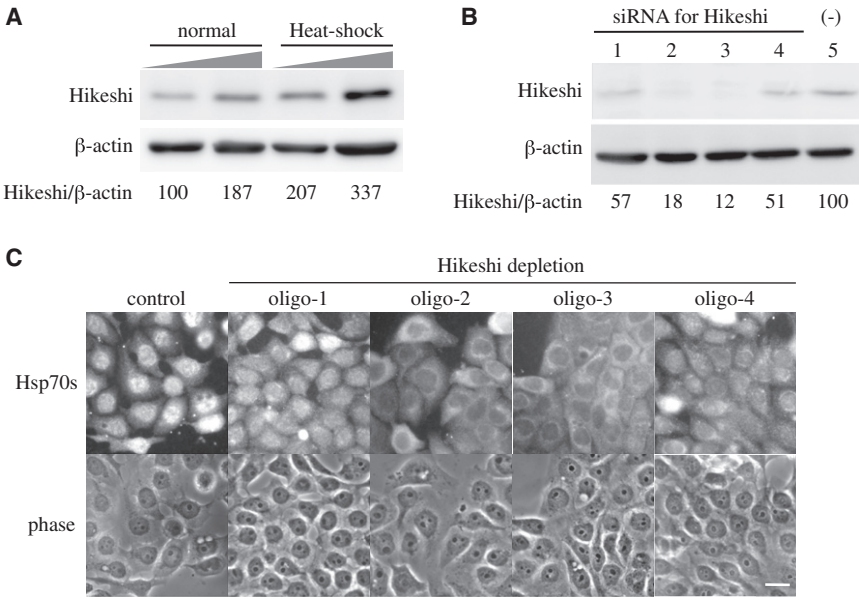


Figure 3. Hikeshi Is Essential for the Nuclear Import of Hsp70s under Heat Shock Conditions in Living Cells

(A) Protein expression of Hikeshi is upregulated under heat shock condition. Extracts of HeLa cells (12.5 or 25 μg) with or without heat shock treatment were separated by SDS-PAGE and then subjected to western blotting. Hikeshi was detected using anti-Hikeshi rat serum. β-actin was used as the loading control. The intensities of each Hikeshi and β-actin signal were measured, and the normalized Hikeshi/β-actin signal ratios are shown.

(B) HeLa cells were transfected with (lanes 1–4) or without (lane 5) four different siRNA duplexes (numbers 1–4) for Hikeshi. The intensities of each Hikeshi and β-actin (loading control) signal were measured, and the normalized Hikeshi/β-actin signal ratios are shown. Hikeshi protein expression was reduced down to ~10%–20% by transfection with siRNA duplex number 2 (lane 2) or 3 (lane 3).

(C) Immunofluorescence of endogenous Hsp70s in heat-shocked HeLa cells with or without siRNA treatment for Hikeshi. The nuclear accumulation of Hsp70s in response to heat shock is inhibited in HeLa cells transfected with siRNA duplex number 2 or 3. Scale bar, 20 μm.

Hikeshi Is Essential for the Heat Shock-Induced Nuclear Import of Hsp70s in Living Cells

In human cells, the expression of the Hikeshi protein increased 2- to 3-fold with heat shock treatment (Figure 3A). To analyze the effect of Hikeshi on the nuclear import of Hsp70s in living cells, Hikeshi was depleted from cells using four different siRNAs. Quantitative real-time PCR analysis (data not shown) showed that the RNA expression levels of Hikeshi were reduced to ~10% by siRNA treatment using oligo-2 or -3, which is consistent with protein expression levels examined by western blotting (reduced to 10% –20%, Figure 3B). This level of Hikeshi depletion strongly inhibited the heat shock-induced nuclear accumulation of Hsp70s (Figure 3C). These results show a crucial role for Hikeshi in the heat shock-induced nuclear import of Hsp70s in living cells.

The above results, seen both in the reconstituted transport and in living cells, confirm that Hikeshi is essential for the heat shock-induced nuclear import of Hsp70s. We next asked whether Hikeshi indeed functions as a nuclear import carrier for Hsp70s that operate in response to heat shock. If so, Hikeshi must fulfill at least two criteria. First, cargo binding and cargo release must be regulated to allow the cargo to accumulate in one compartment from another against a chemical concentration gradient (i.e., for nuclear import, a cargo must bind to a carrier in the cytoplasm, whereas it must be released in the nucleus). Second, Hikeshi must bind to FG-Nups and translocate through NPCs on its own, as all known nuclear transport carriers do.

Binding of Hikeshi to Hsp70s Is Regulated by Cochaperones

To determine whether Hikeshi fulfills the first criterion, we examined the interactions between Hikeshi and Hsp70s. A pull-down assay showed that Hsp70s from importin-depleted cytosol bound efficiently to GST-Hikeshi only when ATP was present (Figure S2A). Furthermore, Hsp70s were precipitated with phenyl-Sepharose, which mimics the hydrophobic property of NPC components (Ribbeck and Görlich, 2002), only when recombinant Hikeshi protein was added in the presence of ATP (Figure 4A). Despite the evidence that Hikeshi binds (Figures 4A and S2A) to and supports the nuclear import of Hsp70s in a crude extract (Figure 2E), Hikeshi is unlikely to bind recombinant Hsp70s properly in the absence of additional soluble factors and ATP because it cannot support the nuclear import of Hsc70 without the importin-depleted fraction and ATP (Figure 4B).

To search for additional factors that cooperate with Hsp70s in binding to Hikeshi, we next dissected the importin-depleted fraction biochemically and identified the Hsp110 family, which functions as a nucleotide exchange factor of Hsp70s (Kampinga and Craig, 2010; Mayer, 2010), as a candidate (data not shown). Strikingly, when Hsc70 was preincubated with GST-Hsp110 in the presence of ATP and then incubated with permeabilized cells in the presence of Hikeshi after the absorption of Hsp110, the preincubated Hsc70 accumulated efficiently in the nucleus (Figure 4C). In contrast, when Hsc70 was preincubated with GST-Hsp110 in the presence of ADP instead of ATP, it failed to accumulate in the nucleus, even in the presence of Hikeshi (Figure 4C). Furthermore, an ATPase-deficient point-mutated Hsc70 (D199S) (Wilbanks et al., 1994) migrated into the nucleus in the presence of recombinant Hikeshi alone and ATP (Figure 4D). These results indicate that Hikeshi can preferentially bind to and carry the ATP-form Hsp70s into the nucleus. The results also explain why ATP is required for Hikeshi to bind Hsp70s in crude extract.

To further understand the importance of the nucleotide state of Hsp70s (ATP versus ADP) for binding Hikeshi, we examined the effect of Hsp40, which stimulates the ATPase activity of Hsp70s

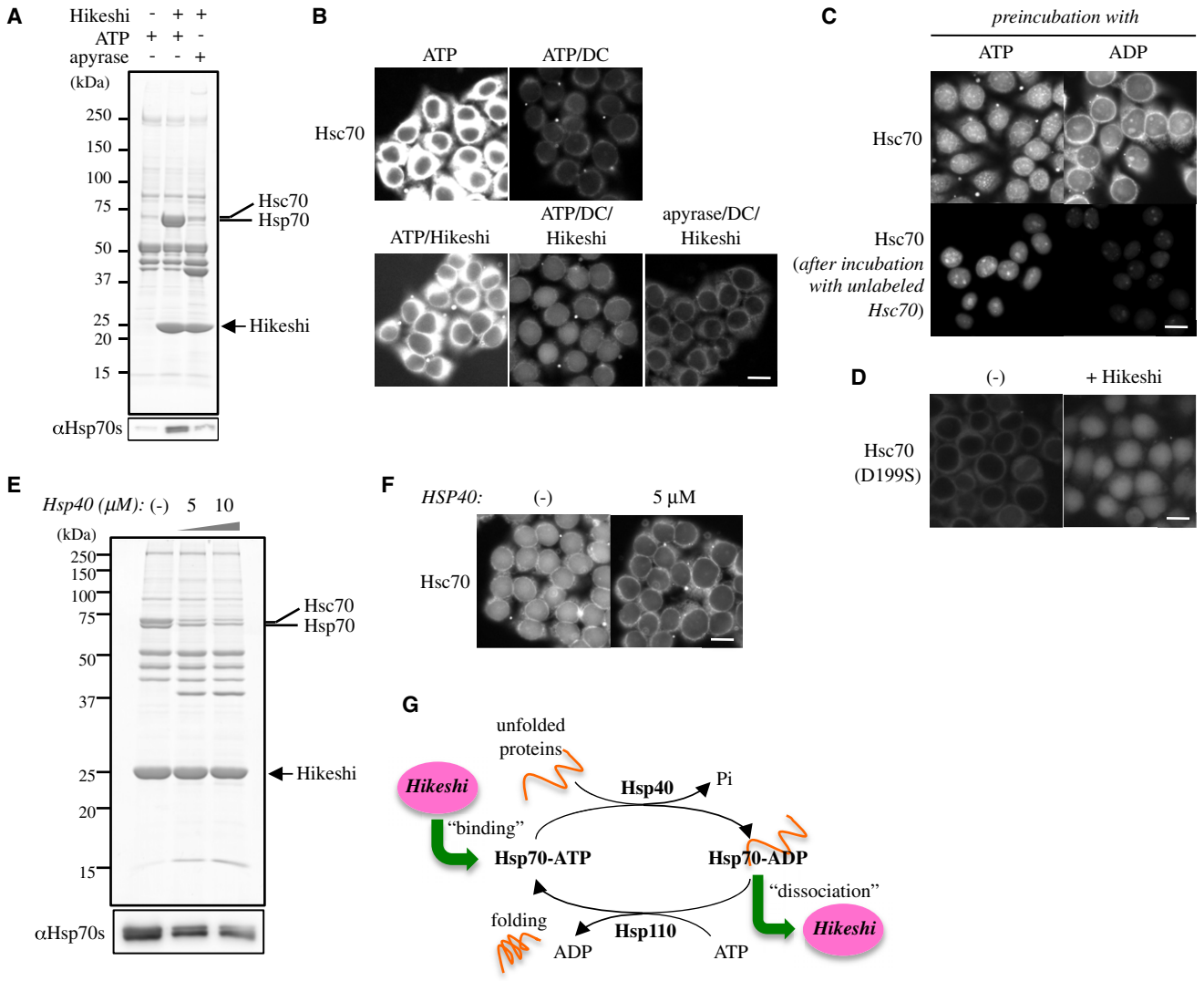


Figure 4. Hikeshi Carries ATP-Form Hsc70, but Not the ADP Form, into the Nucleus

(A) Hsp70s bind efficiently to Hikeshi in the presence of cytosol and ATP. Heat-shocked HeLa extracts were incubated with or without recombinant Hikeshi proteins and ATP and then subjected to pull-down assays with phenyl-Sepharose. Hsp70s was detected by immunoblotting with anti-Hsp70s antibodies.

(B) Nuclear import of Hsc70 is supported in the presence of Hikeshi, DC, and ATP. Scale bar, 20 μm.

(C) Hikeshi efficiently transports ATP-form Hsc70 into the nucleus of the permeabilized cells. GFP-Hikeshi was preincubated with Hsp110 and ATP or ADP and then incubated with the permeabilized cells for 20 min at 30°C (top) after removing Hsp110. (Bottom) Permeabilized cells were incubated with unlabeled recombinant Hsc70 after the transport assay to eliminate nonspecific binding signals. Scale bar, 20 μm.

(D) An ATPase-deficient Hsc70 mutant (D199S) is imported efficiently into the nucleus of the permeabilized cells by Hikeshi alone in the presence of ATP. Scale bar, 20 μm.

(E) Hsp40 inhibits the binding of Hikeshi and Hsp70s. Pull-down assays were performed as indicated in Figure 4A, except that recombinant Hsp40 proteins were added to the extracts.

(F) Inhibitory effect of Hsp40 on the nuclear import of Hsc70. The addition of recombinant Hsp40 proteins to DC inhibited the nuclear import of GFP-Hsc70 in the permeabilized cells. Scale bar, 20 μm.

(G) Binding and release of Hikeshi to Hsp70s is regulated by cochaperones that modulate the nucleotide form of Hsp70s.

See also Figure S2.

(Kampinga and Craig, 2010). The addition of Hsp40 disrupted the binding of Hsp70s with Hikeshi and potently inhibited its nuclear import (Figures 4E and 4F). These results indicate that Hikeshi preferentially binds to the ATP-form Hsp70s, but not to the ADP-form Hsp70s.

In summary, our results indicate that the binding and release of Hikeshi to Hsp70s is regulated by cochaperones that modulate the nucleotide form of Hsp70s, which allows Hsp70s to accumulate in the nucleus from the cytoplasm against a chemical concentration gradient (see Figure 4G and Discussion). Thus,

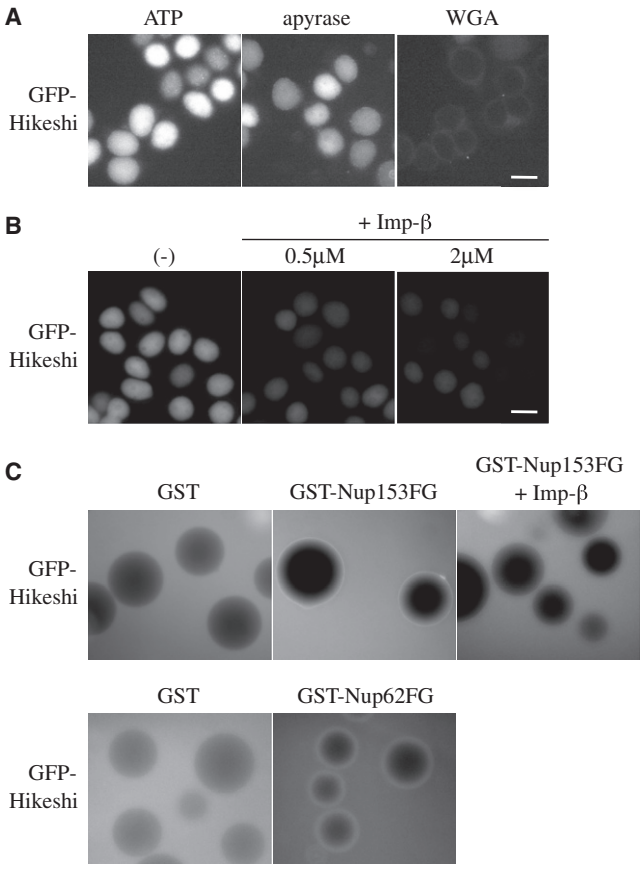


Figure 5. Hikeshi Translocates through Nuclear Pores via a Direct Interaction with Nups

(A) GFP-Hikeshi translocates into the nucleus of permeabilized cells without the addition of other soluble proteins in an ATP-independent manner. Nuclear migration of GFP-Hikeshi is inhibited in the presence of WGA, demonstrating that the nuclear migration of GFP-Hikeshi is not the result of passive diffusion. Scale bar, 20 μm.

(B) The nuclear migration of Hikeshi is competitively inhibited by the addition of excess importin β. Recombinant importin β proteins were added at the indicated concentration in the import reactions of GFP-Hikeshi. Scale bar, 20 μm.

(C) Hikeshi binds directly to FG-Nups. Glutathione Sepharose beads bound to GST-Nup153FG, GST-Nup62FG, or GST were incubated with GFP-Hikeshi. The interaction between Hikeshi and FG-Nups was competitively inhibited by the addition of importin β.

See also Figure S3.

we conclude that Hikeshi fulfills the first criterion of a nuclear import carrier.

Hikeshi Binds to FG-Nups and Translocates through NPCs

To determine whether Hikeshi fulfilled the second criterion, we next examined the ability of Hikeshi to translocate through NPCs on its own as well as its ability to bind to FG-Nups. When recombinant GFP-Hikeshi was incubated with permeabilized cells, it accumulated efficiently in the nucleus, even in the absence of soluble factors and ATP (Figure 5A). Addition of wheat germ agglutinin (WGA) and importin β inhibited the nuclear migration of Hikeshi (Figures 5A and 5B), demonstrating that this nuclear migration was not a result of passive diffusion, but was achieved through a specific interaction with NPC components like importin β. Furthermore, Hikeshi interacted with FG-Nups, such as Nup62 and Nup153, in Bead Halo assays (Patel and Rexach, 2008), which were also inhibited by excess importin β (Figures 5C and S3). These results show that Hikeshi fulfills the second criterion of a nuclear transport carrier, which is direct binding to FG-Nups and the ability to translocate through NPCs on its own.

Because Hikeshi fulfills two criteria of a nuclear import carrier, we conclude that Hikeshi is indeed a nuclear import carrier of Hsp70s that operates in response to heat shock.

Hikeshi Is Required for Cell Survival after Heat Shock Stress

We next investigated the role of Hikeshi in cellular physiology. Depletion of Hikeshi had no apparent effect on cell growth under normal conditions. However, when the viability of Hikeshi-depleted cells after heat shock treatment at several different temperatures was examined, we found that the cell viability was significantly reduced at all temperatures examined (Figure S4A). These results show Hikeshi's critical role in the protection of cells from heat shock stress damage.

We further examined the effect of Hikeshi depletion on cell viability after heat shock stress by using live imaging. Cell images of control and Hikeshi-depleted cells were captured immediately after release from heat shock stress and for the next 24 hr at 1 hr intervals (see Figure 6A and Movies S1 and S2). As shown in Figure 6A, more than 70% of cells started to die (determined by rounding up and detachment of cells from culture plates) after 5 hr of release from heat shock stress, indicating that most Hikeshi-depleted cells died during recovery from the stress damages instead of dying just after the temperature increase. Notably, in contrast to control cells, large populations of Hikeshi-depleted cells were unable to enter mitosis. Additionally, in rare cases, cells that successfully entered mitosis did not complete cell division (see Figure 6A and Figure S4B). Importantly, the reduced cell viability caused by Hikeshi depletion was reversed, at least in part, by expressing Hsc70 tagged with conventional basic NLS before the heat shock treatment (Figure 6B).

Hikeshi Is Required for Attenuation and Reversion of Heat Shock-Induced Nuclear Phenotypes

Hikeshi is a nuclear import carrier that operates in response to heat shock, which is required for survival of cells after heat shock stress. Thus, we asked whether Hikeshi is involved in any of the known stress-induced nuclear phenotypes.

It is well established that heat shock proteins are expressed by the activation of heat shock factor 1 (HSF1) in cells (Voellmy, 2004). The activity of HSF1 is rapidly upregulated during the heat shock response, but HSF1 is restrained soon after release from heat shock stress. The restraint of activated HSF1 during recovery from the stress is essential for cell survival. In Hikeshi-depleted cells, HSF1 was rapidly activated as in normal cells, but the activity of HSF1 was sustained at high levels for a significantly longer period of time as compared with control cells after release from heat shock stress (Figure 7A).

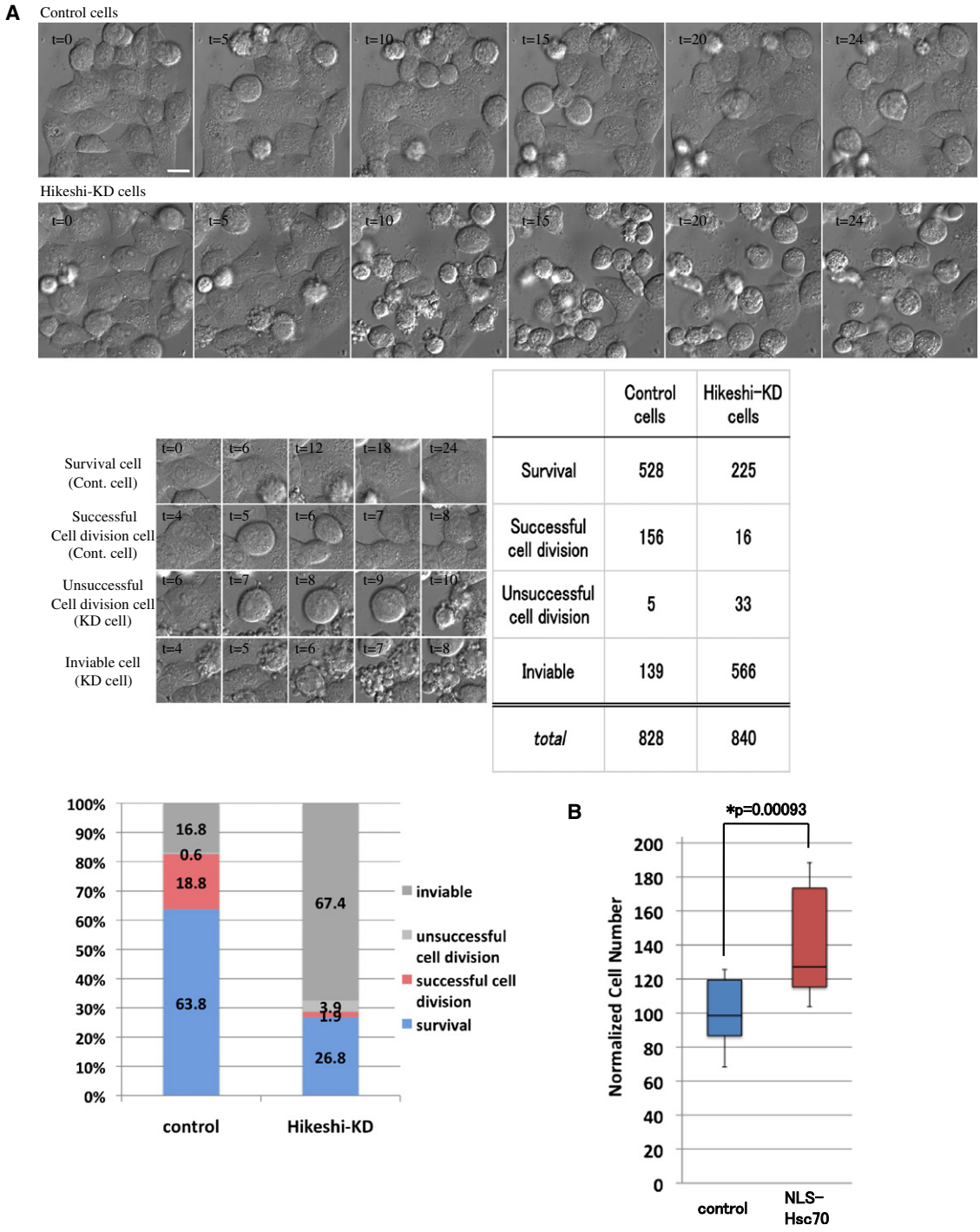


Figure 6. Hikeshi Is Required for Cell Survival after Heat Shock Stress

(A) The viability of Hikeshi-depleted cells decreased significantly after heat shock treatments. Cells treated at 43°C for 1 hr were returned to 37°C. Immediately after the temperature shift-down, live images of cells were captured for 24 hr at 1 hr intervals (see Movies S1 and S2). The number of viable cells was counted and is shown in the graph. Typical examples of cell phenotypes indicated in the table and graph are shown in the middle panels. Scale bar, 15 μm.

(B) Expression of conventional, basic NLS-tagged Hsc70 protein protects cell damage against heat shock stress in Hikeshi-depleted cells. Plasmid-expressing enhanced green fluorescent protein EGFP (control) or EGFP-NLS_{SV40}-Hsc70 was transfected into the Hikeshi-siRNA-treated cells using Lipofectamin 2000 (Invitrogen). At 24 hr after transfection with EGFP or EGFP-NLS_{SV40}-Hsc70, HeLa cells were treated at 43°C for 1 hr and then returned to 37°C, followed by 24 hr incubation in which the number of viable cells was counted (n = 6 each) and normalized to a mean value of control cells. p values calculated using Student's t test. See also Figure S4 and Movies S1 and S2.

In human cells, in response to various cellular stresses, HSF1 rapidly forms nuclear foci, known as nuclear stress granules (nSGs), which originate from the transcription of large pericentromeric-heterochromatic blocks triggered by the activation of HSF1 (Cotto et al., 1997). The kinetics of nSGs formation parallels the transient induction of heat shock proteins (Cotto et al., 1997; Shi et al., 1998). nSGs rapidly appear in Hikeshi-depleted cells, similar to control cells upon heat shock treatment, confirming that Hikeshi depletion does not affect the ability of cells to respond to heat shock. However, nSGs persisted for much longer in Hikeshi-depleted cells than in control cells after a temperature downshift (Figures 7B and 7C), which is consistent with the sustained higher expression of Hsp70 mRNA (Figure 7A). These results show that the depletion of Hikeshi significantly delays the attenuation of the heat shock response.

The nucleolus is a nuclear compartment that exhibits dramatic changes in its organization and composition during heat shock stress, thus affecting DNA metabolism and ribosome biogenesis. One documented phenomenon is the depopulation of proteins, whereby major nucleolar proteins such as nucleolin and nucleophosmin disperse into the nucleoplasm during heat shock stress. Release from the stress rapidly reverts this phenomenon, and dispersed nucleolar proteins reaccumulate in the nucleolus within an hour after release from heat shock stress in normal cells. However, in the Hikeshi-depleted cells, this reaccumulation of nucleolin was strongly inhibited (Figure 7D).

In summary, we conclude that the nuclear import carrier identified in this study, which mediates heat shock-induced nuclear accumulation of Hsp70s, is required to protect cells from heat shock damages and is required in the attenuation and reversion of multiple heat shock-induced nuclear phenotypes. These functions are reminiscent of Hikeshi. The Hikeshi-mediated nuclear import of Hsp70s is required, at least in part, for protection of cells from heat shock damage, which in turn shows that Hsp70 counteracts the damage by acting inside of the nucleus.

DISCUSSION

During heat shock stress, conventional nuclear import pathways mediated by importin β family carriers are downregulated, whereas the nuclear import pathway identified in this study is activated, as in the model depicted in Figure 7E. Hikeshi, an evolutionarily conserved protein with unknown function that does not belong to the importin β family, mediates this nuclear import pathway. Depletion experiments revealed that Hikeshi is involved in attenuation and reversion of multiple heat shock-induced nuclear phenotypes and therefore plays an important role in cell survival under protein-damaging stresses.

Hikeshi Is a Nuclear Import Carrier for Hsp70s Possessing NPC Passage Activity

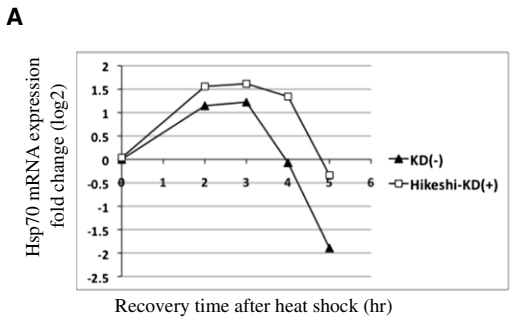
The NPC is composed of multiple copies of about 30 different Nups, many of which contain FG repeat domains (FG-Nups) that are unstructured or natively unfolded (Denning et al., 2003) and are thought to contribute to the highly selective permeability of the NPC (Strambio-De-Castillia et al., 2010). In fact, facilitated selective transport requires specific interactions of the import/export complexes with these hydrophobic clusters for rapid NPC passage (Stewart, 2007). Hikeshi, like the importin β family carriers, interacts weakly but significantly with the FG-Nups examined (Figures 5C, S3C, and S3D) and is capable of translocating through the NPC of permeabilized cells without the addition of soluble factors (Figures 5A and 5B). Hikeshi also binds directly and efficiently to phenyl-Sepharose under low-salt conditions (Figures S3A and S3B), like importin β family carriers. All of these interactions are inhibited competitively by the addition of excess importin β (Figures 5B and 5C), showing that Hikeshi is a soluble protein possessing hydrophobic properties similar to importin β. Importantly, Hsp70s are efficiently enriched by phenyl-Sepharose, which mimics the nature of FG-Nups and accumulates in the nucleus of permeabilized cells, depending on the presence of Hikeshi (Figures 2 and 4).

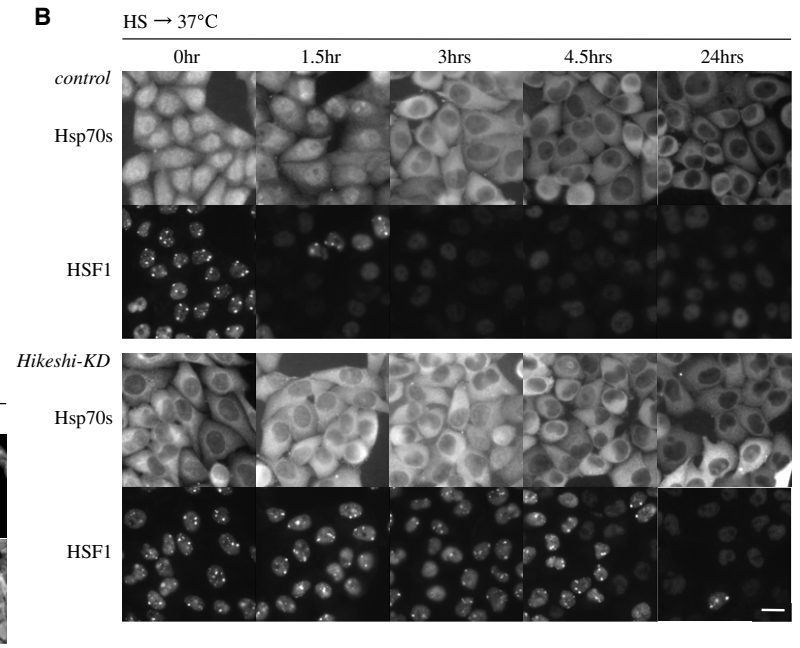
Cochaperones Regulating the Hsp70s ATPase Cycle Are Important for the Hikeshi-Mediated Nuclear Import of Hsp70s

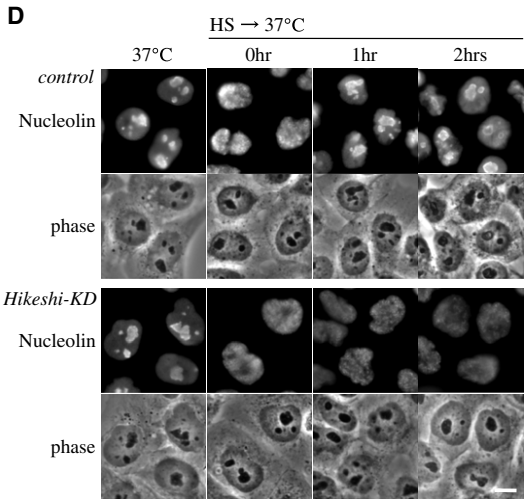
Nucleocytoplasmic transport carriers recognize their cargo molecules in one compartment, which allows them to translocate through the NPC and release the cargo molecules at the destination compartment, resulting in cargo accumulating in one compartment from the other against a chemical concentration gradient. Thus, the transport process, in which cargo binding and release need to be regulated, is usually coupled with the input of metabolic energy. In the transport pathways mediated by importin β family carriers, the Ran GTPase system guarantees the directionality of transport and is thought to be the sole energy source of importin β family nuclear transport pathways. The importin β family carriers, which are RanGTP-binding proteins, sense the concentration of RanGTP across the nuclear envelope and bind or release the cargo in the appropriate compartment.

Hikeshi is not a RanGTP-binding protein and seems unlikely to use the Ran GTPase system during the mediation of import. Instead, Hikeshi binds preferentially to ATP-form Hsp70s, but not to the ADP-form (Figure 4). Therefore, we predicted that the ATPase cycle of Hsp70s is involved in its import. Like all nucleocytoplasmic transport pathways mediated by known carriers (Kose et al., 1997, 1999; Nakielny and Dreyfuss, 1998; Nachury and Weis, 1999), the NPC passage of Hikeshi itself is presumed to be energy independent (Figure 5A). Thus, the input of metabolic energy in the import of Hsp70s supplied through the ATPase cycle of Hsp70s could be consumed for the nuclear accumulation of Hsp70s against a chemical gradient. Hikeshi recognizes the ATP-form Hsp70s in the cytoplasm, carries them into the nucleus, and then releases the Hsp70s in the nucleus where the ATP-form Hsp70s are converted into the ADP form.

The ATPase cycle of Hsp70s is regulated by cochaperones. We demonstrated that Hsp110 is required for Hikeshi to bind to and support the import of Hsp70s (Figure 4C). We also demonstrated that Hsp40 inhibits Hikeshi from binding Hsp70s and mediating their import (Figures 4E and 4F). Therefore, it is likely that Hikeshi recognizes the ATP-form Hsp70s, which are substrate free or supplied after conversion from the ADP-form Hsp70s by NEFs, such as Hsp110s. The ATP-form Hsp70s dissociate from Hikeshi with the aid of the Hsp40 family, which



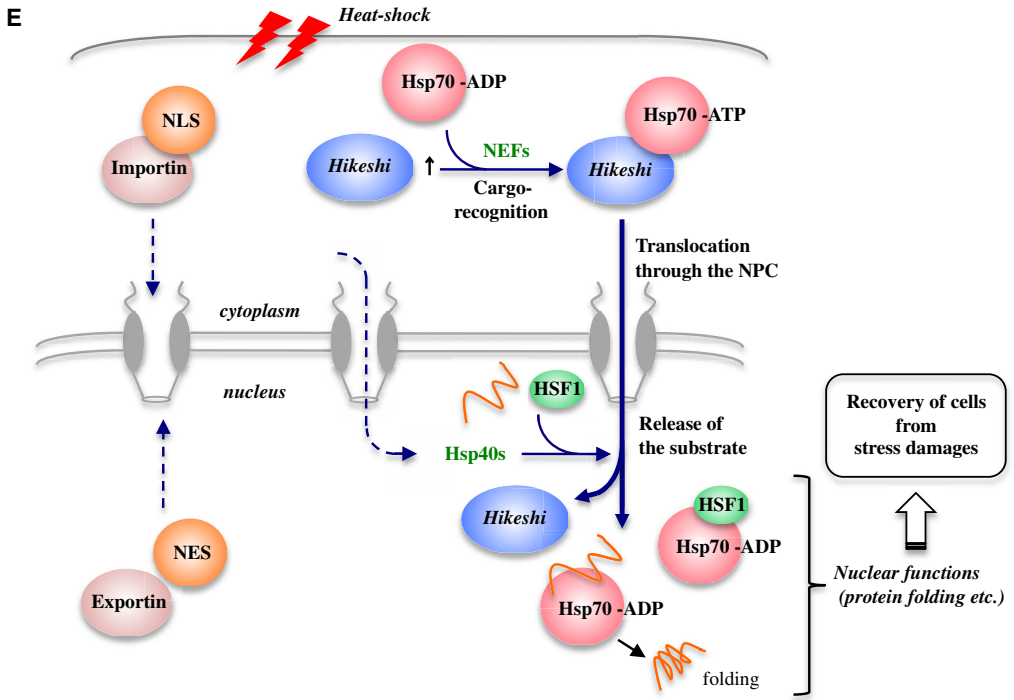




C

43°C → 37°C (hr)	Hikeshi-KD (−)		Hikeshi-KD (+)	
0	94	(80/85)	95	(77/81)
1.5	29	(29/101)	92	(61/66)
3	0	(0/95)	82	(77/94)
4.5	0	(0/83)	63	(70/111)
24	0	(0/77)	4	(3/77)

% (NSGs-positive cells/total cells)



allows Hikeshi to bind native or nonnative client proteins and function as a molecular chaperone in the nucleus (see Figures 4G and 7E).

The complex of Hikeshi and the ATP-form Hsp70s could assemble in the cytoplasm and then translocate into the nucleus through NPCs via the NPC passage activity of Hikeshi. Although many J domain-containing proteins exist in various compartments, human DNAJB1 (Hsp40) is markedly induced in response to heat shock, accumulates in the nucleus and nucleolus with a time course similar to Hsp70s, and colocalizes well with Hsp70s (Hattori et al., 1993). Therefore, such J domain-containing cochaperones could conceivably contribute to dissociating Hsp70s from Hikeshi within the nucleus (see Figure 7E). Because many different cochaperones in cells are involved in either nucleotide exchange or ATP hydrolysis of Hsp70s, it is also possible that other groups of cochaperones participate in driving the import of Hsp70s mediated by Hikeshi.

Switching the Nuclear Transport Pathway under Normal and Stress Conditions

Several cellular stresses induce the modulation of nucleocytoplasmic transport. Such stress signaling is often mediated by the activation of the mitogen-activated protein kinases (MAPK), containing MEK, ERK, and p38, and also induces changes in the cellular distribution of transport factors and efficiency of nucleocytoplasmic transport (Czubryt et al., 2000; Kelley and Paschal, 2007; Kodiha et al., 2009; Kosako et al., 2009). This is similar to what has been demonstrated for Ran, although target molecules of these kinases are currently unknown.

The present study showed that a Ran-independent import pathway mediated by Hikeshi, instead of the conventional Ran-dependent nucleocytoplasmic pathways, is activated in response to heat shock. At least in the reconstituted transport assay that mimics the heat shock-induced transport, addition of the kinase inhibitor staurosporine did not affect Hikeshi-mediated transport of Hsc70 (see Figure S2B), indicating that the transport activation cannot be simply explained by phosphorylation of carrier or cargo molecules. How the balance between conventional and stress-dependent transport in response to several stimuli is achieved in cells is an important question for future research.

Physiological Significance of the Hikeshi-Mediated Nuclear Import Pathway

Hikeshi-depleted cells show reduced cell viability after heat shock stress and retardation of recovery from heat shock-induced nuclear phenotypes (Figures 6A and 7). Although we cannot deny the possibility that Hikeshi has other cargo molecules besides Hsp70s, the reduced viability of Hikeshi-depleted cells can be explained, in part, as a defect in the nuclear function of molecular chaperone Hsp70s because the expression of NLS-tagged Hsp70 before heat shock treatment can partly rescue the viability (Figure 6B). The Hsp70 chaperone machinery functions in protein protection and the recovery from heat-induced protein denaturation in both compartments of the cytoplasm and nucleus in mammalian cells (Michels et al., 1997). Thus, it seems reasonable to assume that protection against and recovery from heat-induced protein damage that occurs within the nucleus would be very difficult in Hikeshi-depleted cells.

We observed that the reversion of multiple heat shock-induced nuclear phenotypes, which are accompanied by alternations in nuclear functions and structures such as the activation of HSF1 (Figure 7A), formation of nSG (Figures 7B and 7C), and depopulation of nucleolar proteins (Figure 7D) during heat shock stress, is severely perturbed in Hikeshi-depleted cells. Although the mechanism of how these heat shock-induced nuclear phenotypes are reverted upon release from stress is currently unknown, there is compelling evidence that heat shock proteins function in the negative feedback regulation of HSF1, presumably linking the balance between chaperons and unfolded proteins in the cells (Voellmy, 2004). Hsp70 associates directly with the transactivation domain of HSF1 and suppresses HSF1 transcriptional activity, which occurs during heat shock response attenuation (Shi et al., 1998). The impaired negative regulation of HSF1 activity in Hikeshi-depleted cells is likely caused, at least in part, by a nuclear import defect of Hsp70s. In any case, defects in the reversion of the heat shock-induced nuclear phenotypes observed in Hikeshi-depleted cells would cause high cellular susceptibility to protein damage from heat shock stress and persistent heat shock-induced nuclear phenotypes, leading to lethal effects for cells. Further analysis of the Hikeshi-mediated nuclear transport pathway identified in the present study would reveal a molecular basis for reverting stress damages caused in the nucleus.

EXPERIMENTAL PROCEDURES

Fractionation of Cell Extracts from Heat-Shocked HeLa-S3 Cells

HeLa-S3 suspension cultured cells were incubated at 43°C for 1 hr with pre-warmed RPMI-1640/5% fetal bovine serum (FBS)/20 mM HEPES (pH 7.3). Heat-shocked cells were resuspended in lysis buffer (50 mM Tris-HCl [pH 7.3], 50 mM NaCl, 5 mM magnesium acetate, and 2 mM DTT) containing 1 mM phenylmethylsulfonyl fluoride (PMSF), 20 μM cytochalasin B, and

Figure 7. Hikeshi Is Required for Attenuation and Reversion of Multiple Heat Shock-Induced Nuclear Phenotypes

(A) Attenuation of the expression of Hsp70 mRNA is suppressed in Hikeshi-depleted cells. After heat shock treatment at 43°C for 1 hr, HeLa cells were returned to 37°C. The expression of Hsp70 mRNA was determined using quantitative real-time PCR.

(B) nSG formation was sustained in Hikeshi-depleted cell nuclei compared with control cells. Heat treatment was performed as in (A). nSGs and Hsp70s were detected by indirect immunofluorescence with anti-HSF1 and anti-Hsp70s antibodies, respectively. Scale bar, 20 μm.

(C) The number of nSG-containing cells counted is indicated.

(D) Relocalization of nucleolin to the nucleolus was suppressed in Hikeshi-depleted cell nuclei as compared with control cells. Heat shock treatment was performed as in (A). Nucleolin was detected by indirect immunofluorescence with anti-nucleolin antibodies. Scale bar, 10 μm.

(E) Model of the nuclear import and function of Hsp70s. In the cytoplasm, Hikeshi, a nuclear import carrier, recognizes ATP-form Hsp70s. The import complex of Hikeshi and ATP-form Hsp70s then translocates into the nucleus through NPCs via the NPC passage activity of Hikeshi. In the nucleus, the ATP-form Hsp70s dissociates from Hikeshi and binds native or nonnative client proteins and functions as a molecular chaperone in the nucleus; this is followed by ATP hydrolysis, which is stimulated by J domain-containing cochaperones, such as the Hsp40 family.

1 μg/ml each of protease inhibitors (aprotinin, pepstatin, and leupeptin) and then lysed using a homogenizer. Cell extracts were rotated with phenyl-Sepharose low substitution (GE Healthcare) equilibrated with lysis buffer. The bound materials were eluted with 50% ethylene glycol in 50 mM Tris-HCl (pH 7.3), 2 mM DTT, and 1 μg/ml each of protease inhibitors and then sequentially applied to a MonoQ column (GE Healthcare) equilibrated with buffer A (50 mM Tris-HCl [pH 7.3], 50 mM NaCl, and 1 mM DTT), a CHT-II column (Bio-Rad), and then a MonoQ column equilibrated with start buffer (50 mM Tris-HCl [pH 8.3], 50 mM NaCl, and 1 mM DTT). In the final MonoQ column, bound proteins were eluted using a linear gradient of 50–200 mM NaCl. Each fraction was equilibrated with transport buffer (TB: 20 mM HEPES [pH 7.3], 110 mM potassium acetate, 2 mM magnesium acetate, 5 mM sodium acetate, 1 mM EGTA, and 1 mM DTT) followed by a concentration using an Amicon Ultra-4 (10 k MWCO; Millipore).

Plasmids

For protein expression in *E. coli*, all genes were cloned into pGEX (GE Healthcare) or pQE (QIAGEN) vectors after PCR using synthetic primers with appropriate restriction sites or the Gateway system (Invitrogen). The human Hikeshi (*C11orf73*), *Hsp40* (*DNAJB1*), and *Hsp110* (*HSPA4*) genes were amplified from a HeLa cDNA library by PCR. Hikeshi and Hsp40 were cloned into pGEX6P-1 vector, and GFP-fused Hikeshi was cloned into pGEX6P-2 vector using Gateway technology. Hsc70 (HSPA8), Hsp70 (HSPA1A), or GFP-fused Hsc70 was cloned into the pQE80L vector. To express the EGFP-NLS-Hsc70 protein in culture cells, EGFP-NLS_{SV40}(PPKKKRKVEDP)-Hsc70 was cloned into the pEF5/FRT/V5-DEST vector (Invitrogen) using Gateway technology. Hsp110 with an N-terminal FLAG tag was cloned into pGEX6P-1 vector using Gateway technology. The FG repeats region of human Nup62 (1–300 aa) and rat Nup153 (886–1468 aa) with a C-terminal His tag was cloned into pGEX4T-3 and pGEX6P-1 vector, respectively.

Expression and Purification of Recombinant Proteins

The expression and purification of recombinant GST-fusion proteins were performed as described previously (Kose et al., 1997).

After purification on glutathione-Sepharose and cleavage of GST, Hikeshi was applied to a MonoQ column (GE Healthcare) and eluted by a linear gradient elution of 50–500 mM NaCl in 50 mM Tris-HCl (pH 8.3) and 1 mM DTT. Purified proteins were dialyzed against TB/1 mM DTT and concentrated using an AmiconUltra-4 (Amicon).

His-tagged Hsc70 and Hsp70 were expressed in *E. coli* strain BL21 (DE3) with the addition of 0.1 mM IPTG, followed by incubation for 14 hr at 20°C. Bacteria were harvested by centrifugation and resuspended in lysis buffer (50 mM Tris-HCl [pH 8.0], 300 mM NaCl, 1 mM 2-mercaptoethanol, and 2 mM PMSF). After cell disruption by freeze thaw and sonication, the extract was clarified by centrifugation and incubated with Ni-NTA agarose at 4°C. After washing with lysis buffer containing 10 mM imidazole, recombinant proteins were eluted with buffer I (20 mM HEPES [pH 7.3], 100 mM NaCl, and 1 mM 2-mercaptoethanol) containing 250 mM imidazole and 1 μg/ml each of aprotinin, pepstatin, and leupeptin. The eluate was incubated with ATP-agarose (linked through C-8; Sigma) and equilibrated with buffer II (buffer I containing 5 mM $MgCl_2$) at 4°C, and the binding proteins were eluted with buffer II containing 5 mM ATP and 1 μg/ml each of aprotinin, pepstatin, and leupeptin. The recombinant proteins were further purified on a CHT-II column (Bio-Rad) using a linear gradient elution of 10–250 mM potassium phosphate (pH 7.3) in 100 mM NaCl and 1 mM DTT, dialyzed against TB/1 mM DTT, and concentrated using an AmiconUltra-4 (Amicon). Aliquots were snap frozen in liquid nitrogen and stored at −80°C.

In Vitro Nuclear Transport Assay

Digitonin-permeabilized HeLa-S3 cells were essentially prepared as previously described (Kose et al., 1997). After permeabilizing the HeLa-S3 cells with 20 μg/ml digitonin, the cells were immersed in TB containing 1 mM DTT and 1 μg/ml each of aprotinin, leupeptin, and pepstatin for 5 min on ice. The permeabilized cells were incubated with heat-shocked cell extract containing 0.8 μM His-GFP-Hsc70, 2 μM Hikeshi, and the regenerating system for 20 min at 30°C. For nuclear import reactions (Figure 4C), 3 μM His-GFP-Hsc70 was incubated with 5 μM Hikeshi, 5 μM GST-Hsp110, and 2 mM ATP or ADP for 20 min at room temperature. After depleting the GST-HSPA4 with glutathione Sepharose, the preincubation mixture was incubated with the permeabilized cells for 20 min at 30°C. To eliminate nonspecific binding of His-GFP-Hsc70, these cells were further incubated with nonlabeled recombinant Hsc70 proteins and 1 mM ATP for 10 min at 30°C. The cells were then fixed in 3.7% formaldehyde in TB, and fluorescent proteins were detected using epifluorescence microscopy (Olympus BX51) with a 40×/0.75 N.A. objective. Images were captured using an ORCA-ER camera (Hamamatsu) controlled by MetaVue software (Universal Imaging).

Small Interfering RNA Experiments

Human Hikeshi (C11orf73) small interfering RNAs (siRNAs) were purchased from QIAGEN. The target sequences were as follows: (1) AUCACGAAACCUA AGUUUAAA, (2) CAGCAAGUGGCAGAGGAUAAA, (3) CUCCUAGGAUUUGU CACGAAU, and (4) CUGCUUAGAGACUGAAGCUUA. These siRNA duplexes were transfected twice using Oligofectamine (Invitrogen).

After washing with PBS, the transfected HeLa cells were lysed using SDS-PAGE sample buffer. Eluted proteins were separated on 12.5% polyacrylamide gels, transferred to PVDF membranes, and immunoblotted with primary antibodies and HRP-conjugated secondary antibodies (Bio-Rad) using the ECL technique. Images were recorded using an LAS-3000 Imager (Fuji Film).

Immunofluorescence Microscopy

Cells grown on coverslips were washed with PBS, fixed in 2% formaldehyde/PBS (Polysciences) for 15 min at 37°C, and then incubated with 50 mM glycine/HMK (20 mM HEPES [pH 7.5], 1 mM $MgCl_2$, and 100 mM KCl) for 5 min at room temperature. After permeabilization with 0.5% Triton X-100/HMK and blocking with 3% skim milk/PBS, cells were incubated with primary antibodies for 2 hr at room temperature and detected with fluorescence-labeled secondary antibodies. Fluorescent images were recorded using an Olympus BX51.

Antibodies

Mouse 1H5-1 monoclonal antibodies (specific to Hsc70/Hsp70), mouse anti-β-actin (Sigma, A5441), rat anti-HSF1 (10H8, Santa Cruz Biotechnology), mouse anti-C23 (nucleolin) (sc-17826, Santa Cruz Biotechnology), and rat anti-Hikeshi serum (produced against full-length recombinant human Hikeshi) were used.

Bead Halo Assay

To detect protein interactions, Bead Halo assays were performed as described previously (Patel and Rexach, 2008). GST or GST-Nup was immobilized on glutathione Sepharose beads at 10 μg/μl and subsequently mixed with 0.1 μg GFP-Hikeshi proteins in EHBN buffer (10 mM EDTA, 0.5% 1,6-hexanediol, 10 mg/ml BSA, 125 mM NaCl). Fluorescent proteins were detected immediately using an epifluorescence microscope (Olympus BX51) with a 40×/0.75 N.A. objective. Images were captured with an ORCA-ER camera (Hamamatsu) controlled by MetaVue software (Universal Imaging).

Quantitative Real-Time PCR

Cell lysates were prepared and reverse transcribed to synthesize cDNA using the procedure of the TaqMan Gene Expression Cells-to-CT Kit (Applied Biosystems). The cDNA was amplified using the 7500 Real-Time PCR System and TaqMan Gene Expression Master Mix and TaqMan Gene Expression Assays for the target genes (Hsp70, Hs00359147_s1; GAPDH, 4333764F). GAPDH mRNA levels served as internal controls.

Live-Cell Imaging

HeLa cells were prepared on a glass-base 3.5 cm dish (Iwaki). After heat shock treatment, culture medium was immediately changed to DMEM/5% FBS prewarmed at 37°C. The dish was then set in the MI-IBC live cell CO_2 chamber (Olympus) at 37°C. Images were captured with the DeltaVision RT microscopy system (Olympus) for 24 hr at 1 hr intervals.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Extended Experimental Procedures, four figures, and two movies and can be found with this article online at doi:10.1016/j.cell.2012.02.058.

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